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HIGHLY BRANCHED β-GLUCAN AND PREPARATION METHOD AND USE THEREOF
[Kobunkido β-Gurukan, Sono Seizoho Oyobi Yoto]

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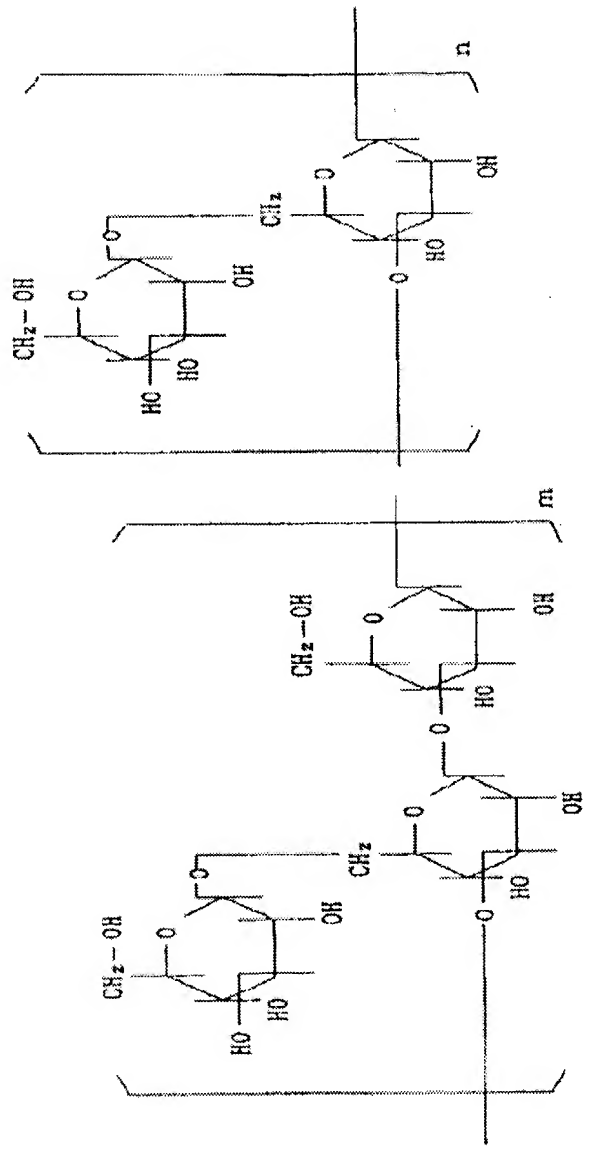
[Claims] /2*

[Claim 1] A highly branched β-glucan that is represented by the following structural formula and that has a number average molecular weight of 10,000 to 5,000,000 (measure by the gel filtration method).

[Chem. 1]



(wherein m is 80 to 30 %, and n is 20 to 70 %).

* Number in the margin indicates pagination in the foreign text.

[Claim 2] A highly branched β-glucan that can be obtained by adding an organic solvent to a culture supernatant of the Aureobasidium pullulans IFO 4466 strain so as to bring about precipitation and that has the following physicochemical characteristics:

(1) it has a number average molecular weight in the range of 10,000 to 5,000,000 (measured by the gel filtration method),

(2) it exhibits an absorption that is specific to the orientation of β-glucosidic linkages at a wavelength of 880 cm^{-1} in an infrared absorption spectrum (according to the KBr method), and

(3) in a ^{13}C NMR spectrum,

(i) it has signals in the vicinity of the δ values of 68 ppm, 86 ppm, and 103 ppm,

(ii) the intensity of the signal at the δ value of 61 ppm is 1.2 to 3.0 times stronger than that of the signal at 60.5 to 60.8 ppm, and

(iii) the intensity of the signal at the δ value of 85.7 ppm is 1.2 to 3.0 times stronger than that of the signal at 86.2 ppm.

[Claim 3] A method for preparing the highly branched β-glucan stated in Claim 1, said method comprising the incubation and culturing of the Aureobasidium pullulans IFO 4466 strain on a liquid medium that contains xylose and Vitamin C as the essential ingredients and collecting the highly branched β-glucan from the obtained culture supernatant.

[Claim 4] A prophylactic medicine for infectious diseases that has the highly branched β-glucan stated in Claim 1 or 2 as an active ingredient.

[Claim 5] An antitumor agent that has the highly branched β-glucan stated in Claim 1 or 2 as an active ingredient. /3

[Detailed Description of the Invention]

[Industrial Field of Application] The present invention pertains to a highly branched β-glucan, a preparation method thereof, and a prophylactic agent for infectious diseases or antitumor agent therewith. The prophylactic agent for infectious diseases or antitumor agent of the present invention are useful as a medicine, food additive, feed additive, etc.

[0002] [Prior Art]

It has been known that genus Aureobasidium sp. produces β-1,3-1,6-D glucans [Acta Chemica Scandinavia, 17, 1351-1356 (1963), Agric. Biol. Chem., 47 (6), 1167-1172 (1983)]. These glucans, however, have phosphoric acid groups, malic acid groups, or sulfonic acid groups attached to them, and these functional groups must be eliminated in order to increase the activities of these glucans. Meanwhile, although glucans that have a large number of branches are also known [Chem. Pharm. Bull., 40, 2215 (1992)], these glucans have a large number of linkages formed between individual main-chain glucoses having no branch, and these glucans also do not have an antitumor activity.

[0003] [Problems that the Invention Intends to Solve]

Focusing attention on the macromolecular polysaccharides produced by the genus Aureobasidium, the present inventors continued researching extensively to obtain a novel and also highly physiologically active β-glucan; as a result, they learned that the Aureobasidium pullulans IFO 4466 strain produced a novel glucan that had highly branched β-glucosidic linkages, that this glucan was a polysaccharide comprised only of glucose that did not bond with phosphoric acid groups, etc., and that it exhibited a high antitumor activity and immunostimulative activity when orally administered. Thus, they achieved the present invention.

[0004] Accordingly, the objective of the present invention is to provide a novel glucan having highly branched β-glucosidic linkages. The present invention also intends to provide a method for producing the novel highly branched β-glucan, using the Aureobasidium pullulans IFO 4466 strain. The present invention further intends to provide an antitumor agent and a prophylactic agent for infectious diseases that contain this novel highly branched β-glucan as an active ingredient.

[0005] [Means of Solving the Problems]

More specifically, as stated in the foregoing, the present inventors focused attention on the polysaccharides produced by the genus Aureobasidium, and they researched extensively on polysaccharide production using various kinds of microorganisms that belonged to the

genus Aureobasidium; as a result, they learned that, in a liquid medium that contained xylose and Vitamin C as essential ingredients, the Aureobasidium pullulans IFO 4466 strain produced, in a high yield, a highly physiologically active β-glucan that had highly branched β-glucosidic linkages.

[0006] The following explains the present invention in further detail. According to "Koza/Shinkin no Bunrui • Dotei (2) [Course on Fungi Classification and Identification (2)]" written by Chikara Morinaga [J. Antibact. Antifung. Agents. 18 (6) 295-297 (1990)], there are 14 kinds and one variant of the Genus Aureobasidium, and these are mostly classified as Aureobasidium pullulans. There are two variants of Aureobasidium pullulans. With respect to their morphological characteristics, their colonies have a smooth surface and often covered with a viscous mass of conidia, and aerial hyphae are sparsely present. The color of the colonies varies and may be light brown, yellow, pink, or black. The hyphae are transparent and often become brown and are thick-walled. The conidiogenous cells are transparent, and they are formed on the hyphae at the tip or middle section, branching off from it. The conidia are synchronously formed densely from the top of the conidiogenous cells by budding. They are single cells that are transparent in color and smooth-walled and have various shapes and sizes. The present invention can use any strains as long as they have been isolated from these Aureobasidium pullulans in nature and purified and subcultured, thus maintaining their character, or as

long as they are strains that are selected from the strains deposited at depository institutions and that can produce the highly branched β-glucan of the present invention,. However, it is preferable to use the Aureobasidium pullulans IFO 4466 deposited at the foundation, Hakko Kenkyusho [Fermentation Research Center] in terms of the yield of the highly branched β-glucan and ease of isolation.

[0007] The medium employed in the present invention is a liquid medium that contains nutrients, such as a carbon source, nitrogen source, phosphor, potassium, magnesium, etc., that are commonly required for microorganism culture. With respect to the carbon source, xylose and Vitamin C, as a minimum, are used as the essential ingredients. In addition to these, glucose and sucrose may also be used as the carbon source. Xylose as the carbon source is used in a quantity of 5 to 150 g/L, preferably 10 to 100 g/L, better yet, 20 to 60 g/L, and Vitamin C is used in a quantity of 0.01 to 100 g/L, preferably 0.1 to 60 g/L, better yet, 0.5 to 20 g/L. With respect to the ratios of the ingredients other than the carbon source, $NaNO_3$ is used in a quantity of 0.5 to 20 g/L, preferably 1 to 10 g/L; K_2HPO_4, 0.05 to 10 g/L, preferably 0.1 to 5 g/L; KH_2PO_4, 0 to 20 g/L, preferably 0.5 to 5 g/L; KCL, 0.1 to 10 g/L, preferably 0.2 to 5 g/L; $MgSO_4 \cdot 7H_2O$, 0.05 to 5.0 g/L, preferably 0.1 to 2.0 g/L; and $FeSO_4 \cdot 7H_2O$, 0 to 5 g/L, preferably 0.005 to 2.0 g/L. Vitamin B_1 may also be added to the liquid medium of the present invention. The culturing is usually conducted at a temperature of 5 to 40° C for 1 to 10 days. The

culturing is preferably conducted under aeration. In this manner, the highly branched β-glucan of the present invention is produced in the culture fluid.

[0008] After the culturing is completed, the culture fluid is subjected to centrifugation, etc., so as to eliminate the fungus body from the culture fluid, and the highly branched β-glucan of the present invention is collected from the supernatant. As the collection method, a method that adds an organic solvent to the culture supernatant so as to precipitate the highly branched β-glucan of the present invention is preferably used. No specific limitation is imposed on the organic solvent here, and alcohol, ketone, nitrile, /4 etc., may be used. More specifically, ethanol, isopropyl alcohol, acetone, actonitrile, etc., may be listed here, but ethanol is especially desirable. In addition to the highly branched β-glucan of the present invention, the obtained product usually contains such impurities as low-molecular compounds, proteins, water-insoluble glucans, etc. When the highly branched β-glucan of the present invention is used as a food additive or feed additive according to the present invention, the aforesaid product may be used as it is or after it is dried.

[0009] However, in the case of using it as an active ingredient in pharmaceutical products, etc., low-molecular compounds are eliminated by dialysis, using a cellulose tube, etc., and proteins are

eliminated by precipitation using an acidic substance, such as trichloroacetic acid, picric acid, etc., or an organic solvent, such as n-butanol or a chloroform solution of n-butanol, as the deproteinizing agent. Furthermore, to the precipitate of the highly branched β-glucan, an alkali aqueous solution of about 0.5 N is added to dissolve this precipitate, and the insoluble glucans are eliminated by precipitation. Only the water-soluble glucan is neutralized with such an acid as acetic acid, citric acid, hydrochloric acid, sulfuric acid, etc., and refined, thereby obtaining the highly branched glucan. The chemical substances used in the process of impurity elimination are eliminated by dialysis, gel permeation, ultrafiltration, etc., thereby obtaining the highly branched β-glucan of the present invention with high purity.

[0010] The highly branched β-glucan thus obtained has the following physicochemical characteristics.

[0011] (1) Constituent monosaccharide

To 50 mg of the aforesaid highly branched β-glucan was added 2 mL of 1N sulfuric acid, and the mixture was heated for 8 hours to hydrolyze it and subsequently reduced with sodium borohydride according to a conventional method, after which it was acetylated with pyridine and acetic anhydride and analyzed by gas chromatography (column: 3 wt % ECNSS-M/Chromosorb W, temperature: 190° C, carrier gas: nitrogen gas, carrier gas flow rate: 3 mL/minute). As a result, it was found that

the specific rotation $[\alpha]_D^{25}$ was +50°, and, since it approximately matched that of D-glucose [The reference value $[\alpha]_D^{25}$ is +52.8° ("Yuki Teisei Bunseki [Organic Qualitative Analysis]" p.276, published by Hirokawa Shoten), it was confirmed that 99 % or more was glucose. Furthermore, the highly branched β-glucan of the present invention was subjected to enzymic decomposition by exo-β-glucanase obtained by refining commercially available quilatase [as transliterated], and the resulting decomposed sugar was analyzed by thin layer chromatography (TLC). Although glucose alone is detected when this refined enzyme acts on laminarin, which is comprised of β-1,3-linked glucose alone, and the resulting decomposed sugar is analyzed by TLC, the decomposed sugar obtained from the highly branched β-glucan of the present invention had the same Rf values as those of glucose and gentiobiose. Moreover, there were two or more gentiobioses to one glucose. Since the decomposition speed by the enzyme was 1/20 of the decomposition speed of laminarin, it was learned that the scission of the main-chain β-1,3 linkages received a steric hindrance by the branched chains. The enzymic decomposition product obtained in this manner and the aforesaid acid decomposed product were subjected to HPLC analysis (column: μBondaSphere-NH_2 5μ 100Å, solvent: 80 % CH_3CN, flow rate: 0.8 mL/mL [sic], detection: by a differential refractometer), thus quantitatively determining the decomposed sugar. As a result, it was learned that the decomposition rate by the enzyme was 1 % or less of

the decomposition rate by the acid, which indicates that the β-glucan was very difficult to decompose with the enzyme.

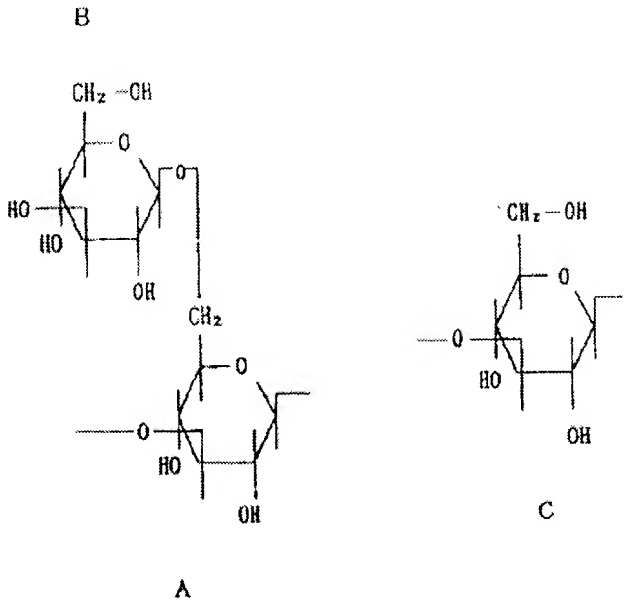
[0012] (2) Glucose linkage and branching degree

Figure 1 shows one example ^{13}C NMR spectrum obtained by dissolving the highly branched β-glucan of the present invention in dimethyl sulfoxide (DMSO)-d_6 at 100° C and by taking a measurement as the temperature was maintained at 100° C. As shown in Fig. 1, there are three signals: (i) peak S_1, which is attributable to the C-6 carbon atom in the glucose residue A shown in Chem. 3, in the δ=68 ppm region; (ii) peak S_2, which is attributable to the C-3 carbon atoms in the aforesaid glucose residue A and glucose residue C shown in Chem. 3, in the δ=86 ppm region; and (iii) S_3, which is attributable to the C-1 carbon atoms in the glucose residues A, B, and C shown in Chem. 3, in the δ=103 ppm region. Based on this, it was determined that, in the highly branched β-glucan of the present invention, the aforesaid B that is linked by means of β1→6 linkage branches off from the main chain comprised of the aforesaid A or C that are linked by means of β1→3 linkage [see Carbohydrate Polymers 2, 135-144 (1982)].

[0013]

[Chem. 2]

/5



[0014] As seen in Fig. 2, which is an enlargement of Fig. 1, while the intensity of signal S_a, which is attributable to the C-6 carbon atom in the glucose residue C, in the δ=60.5 to 60.8 ppm region is one, the intensity of signal S_b, which is attributable to the C-6 carbon atom in the glucose residue B, in the δ=61.0 ppm region is approximately two; therefore, in the highly branched β-glucan of the present invention, there are two branched glucose residues for three main-chain glucose residues.

[0015] Figure 3 shows an enlarged spectrum of the region in which signals attributable to the (β1→3)-linked C-3 carbon atoms are detected. In Fig. 3, a correlation is observed between signal S_c at δ=85.7 ppm and the signals (δ=3.58 and 4.08 ppm) of the H-6 hydrogen atom in the glucose residue A in HSQC-TOCSY ["Erunsuto Nijigen NMR [Ernst's Two-Dimensional NMR]" published by Yoshioka Shobo, p. 589

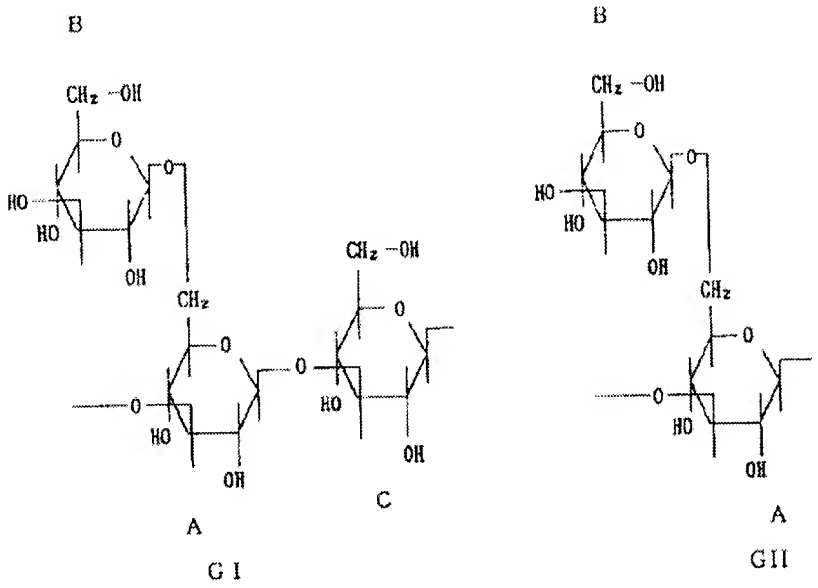
(1991) and "Jikken Kagaku Koza [Experimental Chemistry Course]" edited by the Japan Chemical Association and published by Maruzen, V. 5, p. 133-137 (1991)]; therefore, signal S_c is attributed to the C-3 carbon atom in the glucose residue A [see Carbohydroate [sic] Polymers 2, 135-144 (1982)]. The remaining signal S_d at δ=86.2 ppm is attributed to the C-3 carbon atom in the glucose residue C. Furthermore, the highly branched β-glucan of the present invention does not show a signal corresponding to the C-3 carbon atom at δ=85.9 ppm, which should be observed in such a glucan as scleroglucan or laminarin, that has a unit composed by repeating the glucose residue C as a partial structure; therefore, the glucose residue A always bonds to the C-3 side of the glucose residue C in the highly branched glucan of the present invention. The fact that the intensity ratio of signals S_c and S_d is 2 : 1 shows us that the highly branched β-glucan of the present invention in this example is composed of Unit GI and Unit GII having the structures shown in Chem. 4 and that the abundance ratio of GI and GII is 1 : 1. When, for example, the β-glucan of the present invention is subjected to fractionation at room temperature using DMSO, it can be seen that the β-glucan of the present invention contains DMSO-soluble and DMSO-insoluble components, and, from the area intensity ratio of signals S_a and S_b in the spectrum shown in Fig. 4, the DMSO-soluble component is a β-glucan that has 5 branched glucose residues per 9 main-chain glucose residues, while the DMSO-insoluble component

is a β-glucan that has 3 branched glucose residues per 4 main-chain glucose residues. In other words, the β-glucan of the present invention contains 20 to 70 % of Unit GII.

[0016]

[Chem. 3]

/6



[0017] (3) Infrared absorption spectrum (by a KBr method)

As shown in Fig. 5, an absorption (P) that is characteristic of the orientation of β-glucosidic linkages is observed at a wavelength of 880 cm^{-1}.

[0018] (4) Molecular weight (by gel filtration)

Number average: 10,000 to 5,000,000, preferably 500,000 to 5,000,000

[0019] (5) Color reaction

Morish [as transliterated] reaction, anthrone-sulfuric acid reaction, phenol-sulfuric acid reaction: positive

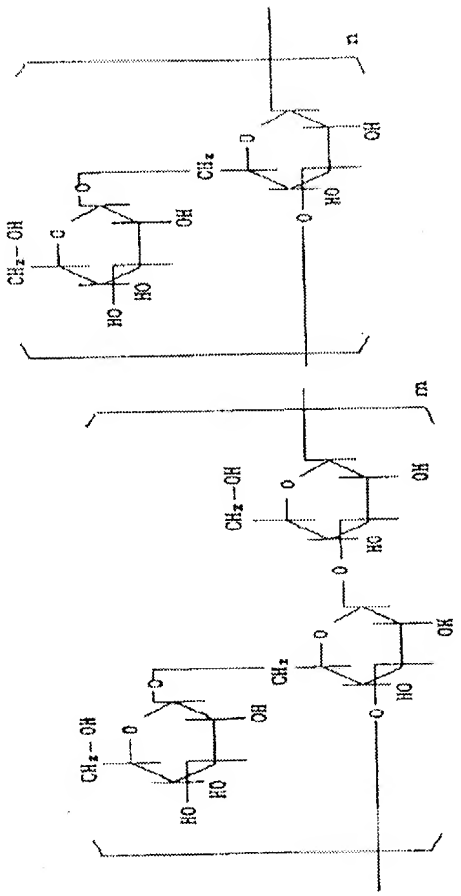
Ninhydrin reaction, biuret reaction: negative

[0020] From the above physicochemical characteristics, the chemical structure of the highly branched β-glucan of the present invention was determined to be as follows.

[0021]

[Chem. 4]

/7



(m is 80 to 30 %, and n is 20 to 70 %.)

[0022] The highly branched β-glucan of the present invention is characterized in that it has β-1,3-linked glucose residues as the main

chain and that these glucose residues have many β-1,6-linked glucose residues that branch off from them; thus, the main chain practically has no linkage between glucose residues having no branches.

[0023] When the antitumor activity of the highly branched β-glucan of the present invention was tested by administering a solution of this glucan in physiological saline orally or parenterally to tumor-bearing mice, the glucan inhibited the exacerbation of the tumor and improved the life-sustaining rate, as demonstrated in working examples. It also had an immunostimulative activity.

[0024] Thus, the highly branched β-glucan of the present invention exhibits an antitumor activity or immunostimulative activity when administered orally or parenterally, and it can be used as a medicine, food additive, or feed additive. In the case of using it as a medicine, it is recommended to administer 100 to 0.1 mg, preferably 30 to 60 mg, per day for an adult a few times a day as an antitumor agent or immunostimulative agent, although the dosage varies depending on symptom, age, sex, etc. Although this highly branched β-glucan can be used alone as a medicine, it can be used, according to a pharmaceutical practice, by formulating it into a compound with a pharmaceutically acceptable diluent and/or other substances that have other pharmacological effects. It can be administered, for example, orally, intravenously, intraperitoneally, or enterically. Therefore, it can be used in forms suitable for these methods of administration—

for example, as a powder, granules, tablets, sugar-coated pills, capsules, pills, a suppository, a suspension, a liquid, an emulsion, an injectable solution, an aerosol, etc. However, if it is used as a tumor-metastasis-preventing agent, oral administration is especially effective.

[0025] To use this glucan as a food additive or feed additive, the aforesaid precipitate or highly branched β-glucan of the present /8 invention may be added as it is to food or feed or may be mixed with a carrier, extender, etc., that are commonly used for these additives and then added to food or feed, thus imparting an antitumor activity or immunostimulative activity to the food or feed to prevent or treat infectious diseases. It is suitable as a feed additive, for example, for cows, pigs, chickens, fish, birds, dogs, cats, etc.

[0026] The following explains the present invention in more concrete terms, referring to working examples.

[Working Example 1]

Preparation of highly branched β-glucan

1. Culture of fungus body

The Aureobasidium pullulans Foundation Hakko Kenkyusho Deposit No. IFO 4466 strain, which had been cultured on a potato dextrose agar slant medium and kept, was inoculated on 300 mL of a liquid medium (pH 5.0 - 6.0, preferably pH 5.5) of the following composition that was placed in a Sakaguchi flask, and aeration/agitation culture was carried out at 20 to 30° C for 2 to 3 days.

Example composition of the medium used in the present invention

Xylose	30 g
Vitamin C	6.0 g
$NaNO_3$	2.5 g
K_2HPO_4	0.4 g
KH_2PO_4	2.0 g
KCl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeSO_4 \cdot 7H_2O$	0.01 g
Vitamin B_1	1 mg
Distilled water	1 L

When glucose or sucrose is used as the sugar, the Aureobasidium pullulans IFO 4466 strain also produces pullulan (α-1,4-1,6-glucan), but, when xylose is used as the sugar, it produces the highly branched β-glucan of the present invention with little production of pullulan (see Table 1).

[0027]

2. Preparation of the highly branched β-glucan of the present invention

Using a centrifugal separator, the fungus body was eliminated from the aforesaid 300 mL culture solution, and, to the culture supernatant thus obtained was added an equal amount of ethanol and stirred for several hours at room temperature. Next, the mixture was centrifuged, and 300 mL of 0.5 N NaOH was added to the obtained precipitate and stirred at room temperature to precipitate the water-insoluble glucan, which was eliminated by centrifugation. The solution from which the insoluble substance had been removed was subjected to dialysis, using

a cellulose tube, etc. If a trace quantity of proteins needs to be eliminated, trichloroacetic acid is added to the coarse precipitate aqueous solution prior to the dialysis so as to precipitate and eliminate the proteins.

Table 1 shows the quantity of the highly branched β-glucan thus produced.

[0028] [Table 1]

Carbon Source	Addition Quantity	Produced Quantity (g/L)	
		Highly branched β-glucan	Pullulan
Glucose	30 g/kL	0.6	0.6
Sucrose	30 g/kL	0.7	0.7
Xylose	30 g/kL	1.2	trace

[0029] When the physicochemical properties of the highly branched β-glucan thus obtained were analyzed, they were found to be identical to those of the highly branched β-glucan shown before.

[0030] [Working Example 2]

The antitumor activity of the highly branched β-glucan obtained in Working Example 1 was determined. To 14 or 7 ICR mice (females, about 30 g) was transplanted 5×10^6 cells of a homograft tumor, Sarcoma 180, under the skin of the inguinal region. No control was imposed on feeding and drinking of water. On the seventh day after the transplantation, 2.5 mg of the highly branched β-glucan of the present invention was dissolved in 1 mL of physiological saline, and this solution was administered into the peritoneal cavity once a day at a dose of 40 mg per 1 kg body weight. On the fifth week after the

transplantation, the tumor was removed and weighed. The results were compared with those of the control group to which only physiological saline was administered. The results are shown in Table 2. As is evident from Table 2, a high antitumor activity was observed when the highly branched β-glucan of the present invention was administered into the peritoneal cavity.

[0031] [Table 2]

Glucan	Dose, mg/kg body weight	Tumor weight	Growth inhibition rate %	Complete involution
Control	0	9.6 ± 7.7	0	0/14
Glucan of this invention	40	0.75 ± 1.1	92	1/7

[0032] [Working Example 3]

The immunostimulative activity of the highly branched β-glucan obtained in Working Example 1 was determined. To ICR mice (females, about 30 g) was intraperitoneally administered a solution of 3 mg of the highly branched β-glucan of the present invention in 1 mL of physiological saline at a dose of 20 mg per 1 kg body weight. No control was imposed on feeding and drinking of water. On the second or third day after the initiation of dosing, the spleen was removed, /9 and its weight and number of cells were measured. Furthermore, the abdominal exudate cells and blood were taken, and the number of the abdominal exudate cells and the number of cells in the blood were measured. Using the abdominal exudate cells, its intake capacity of fluorescence-labeled beads and lysosome-acid phosphatase activity were

measured to determine the phagocytic activity of the abdominal exudate cells. The results were compared to those of the control group, as in Working Example 2. The results are shown in Tables 3 through 6. As seen in Tables 3 through 6, with the intraperitoneal administration of the highly branched β-glucan of the present invention at a dosage of 40 [sic] mg/kg body weight, the weight of the spleen increased to twice that of the non-dosed group (control) by the second day after the administration, and the number of cells also increased to 1.8 times. The number of the abdominal exudate cells and the number of lymphocytes in the blood also increased to 3.4 times and 1.4 times respectively by the third day after the administration. The bead-intake capacity of the macrophages of the abdominal exudate cells increased to 1.2 times, and the phosphatase activity also increased to 2.2 times, thus indicating that the phagocytic activity was increased. Based on these findings, the immunostimulative activity was determined to be enhanced considerably.

[0033] [Table 3]

Effects of the highly branched β-glucan of the present invention on increasing the total weight and number of the cells of pancreas [sic]

Glucan	Dosage mg/kg body weight	Weight		No. of cells	
		2 days later (mg)	3 days later (mg)	2 days later, cells/mg	3 days later, cells/mg
Control	0	133	133	1.8×10^8	1.8×10^8
Glucan of this invention	20	265	214	3.2×10^8	2.6×10^8

[Table 4]

Numbers of abdominal exudate cells and lymphocytes in blood

Glucan	Dosage mg/kg body weight	No. of abdominal exudate cells		No. of lymphocytes in blood	
		2 days later cells/mL	3 days later cells/mL	2 days later, cells/mL	3 days later, cells/mL
Control	0	2.6×10^6	2.6×10^6	2.6×10^6	2.6×10^6
Glucan of this invention	20	6.0×10^6	8.8×10^6	6.7×10^6	6.6×10^6

[Table 5]

Bead-intake capacity in abdominal exudate cells

Glucan	Dosage mg/kg body weight	Intake capacity (%) 3 days later
Control	0	71
Glucan of this invention	20	82

[Table 6]

Lysosome-acid phosphatase activity of macrophages in abdominal exudate cells

Glucan	Dosage mg/kg body weight	Lysosome-acid phosphatase activity (relative activity)	
		2 days later	3 days later
Control	0	1	1
Glucan of this invention	20	1.3	2.2

[0034] [Working Example 4]

To 7 to 8 ICR mice (females, about 30 g) was transplanted 5×10^6 cells of a homograft tumor, Sarcoma 180, under the skin of the abdominal region. Immediately after the transplantation, the highly branched β-glucan-containing physiological saline that was used in Working Example 2 was forcefully administered orally or administered /10

intraperitoneally to the mice. They were allowed to feed and drink water freely. On the fifth week of the glucan dosing, they were weighed, and the tumor was removed and weighed. As in Working Example 2, the comparison with the control group was carried out. The results are shown in Tables 7 through 9.

[0035] [Table 7]

Tumor-growth inhibiting effect of the highly branched β-glucan

Dosing method	Dosage mg/kg body weight	Solid tumor weight (g)	Solid tumor inhibiting rate (%)	No. of total involution	Life-sustaining rate (%)
Control	0	5.23	0	0/8	13
Intra-peritoneal dosing	25	2.56 ± 2.32	51	1/7	86
Oral dosing	90 180	2.03 ± 2.23 6.98 ± 3.46	61 -44	1/7 0/7	71 71

[Table 8]

Number of mice that developed ascites tumor and quantity of collected ascites fluid

Dosing method	Dosage mg/kg body weight	Rate of developing ascites tumor (%)	ascites fluid quantity (average) mL
Control	0	88	21.5
Intraperitoneal dosing	25	14	0.5
Oral dosing	90 180	29 29	15.6 11.6

[Table 9]

Weight increase in Mice

Dosing method	Dosage mg/kg body weight	No. of mice(immediately after transplantation → 35th day)	Weight increase (g)
Control	0	8 → 1	3.3 (1)
Intraperitoneal dosing	25	7 → 6	10.6 (2.4)
Oral dosing	90 180	7 → 5 7 → 5	5.4 (1.6) 5.7 (1.7)

[0036] As seen from Tables 7 through 9, when the highly branched β-glucan of the present invention was administered orally at a dosage of 90 mg/kg body weight, the growth of the solid tumor was inhibited to 61 % in comparison with the non-dosed group (control), and the life-sustaining rate was improved to 71 %, compared to 13 % of the control group. While the ascites tumor-developing rate of the control was 88 %, the present invention could reduce the tumor-developing rate to 29 %, and it could increase the weight to 1.6 times that of the non-dosed group. Judging from the fact that this weight increase was due to the increase of the weight of the spleen, which is involved in immunity, the immunostimulative activity was believed to be extremely enhanced.

[0037] [Working Example 5]

(1) In 10 mL of physiological saline, 5 mg of the highly branched β-glucan obtained in Working Example 1 was dissolved, thus preparing an antitumor agent or immunostimulative agent that could be administered orally.

(2) 5 mg of the highly branched β-glucan obtained in Working Example 1 was mixed with 50 mg of lactose, mannitol, and grape sugar, and the mixture was formed into tablets.

(3) 20 g of the precipitate of the highly branched β-glucan obtained in Working Example 1 was added to 1 kg of cow formula feed, thus enhancing the immunity of cows so as to prevent infectious diseases.

[0038] [Effects of the Invention]

According to the present invention, the novel highly branched β-glucan can be mass-produced at a high yield by simple cultivation. Since the obtained highly branched β-glucan exhibits a high antitumor activity and immunostimulative activity when administered orally, it is effective as a tumor-preventive or curative medicine for humans or as an agent to prevent or cure infectious diseases for livestock, pets, cultured fish, etc. /11

[Brief Explanation of the Drawings]

[Fig. 1] It is a two-dimensional NMR spectrum of the highly branched β-glucan.

[Fig. 2] It is a spectrum obtained by enlarging the two-dimensional NMR shown in Fig. 1.

[Fig. 3] It is a spectrum obtained by enlarging the two-dimensional NMR shown in Fig. 1.

[Fig. 4] It presents NMR spectra of the DMSO-soluble component and insoluble component of the highly branched β-glucan.

[Fig. 5] It shows an infrared absorption spectrum (by the KBr method) of the highly branched β-glucan.

[Fig. 6] It shows the relationship between the dosing of the highly branched β-glucan and the life-sustaining ratio in Working Example 4.

[Explanation of the symbols]

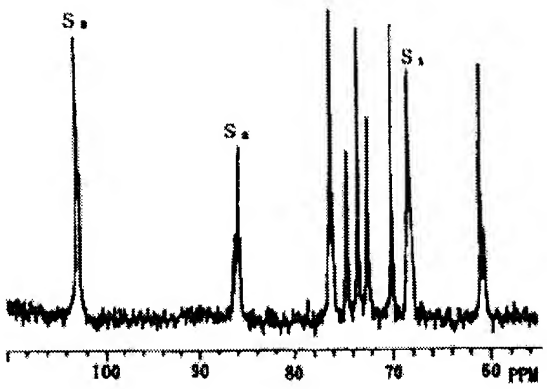
○ Control

◇ Oral administration (90 mg/kg)

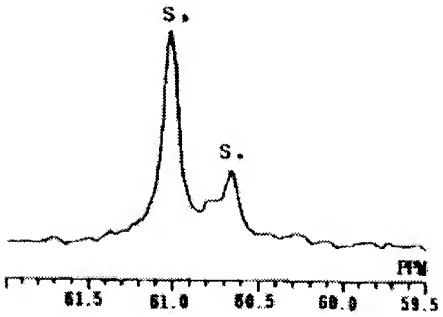
◆ Oral administration (180 mg/kg)

□ Intraperitoneal administration (25 mg/kg)

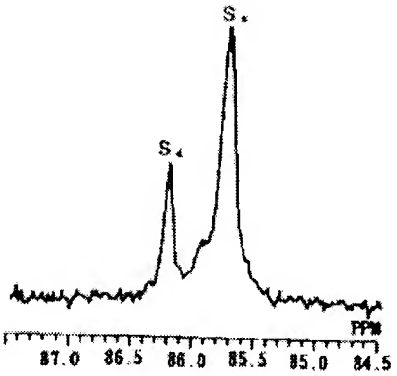
[FIG. 1]



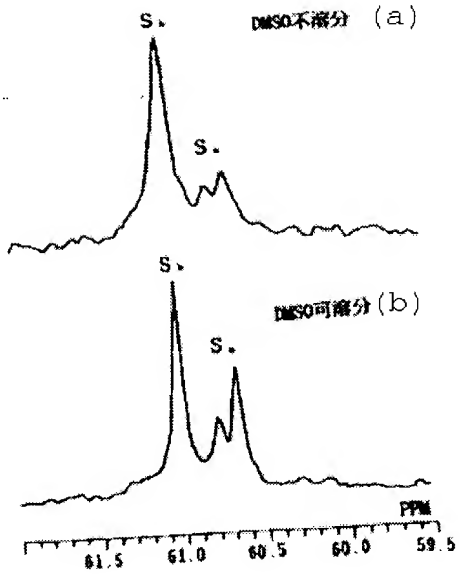
[FIG. 2]



[FIG. 3]

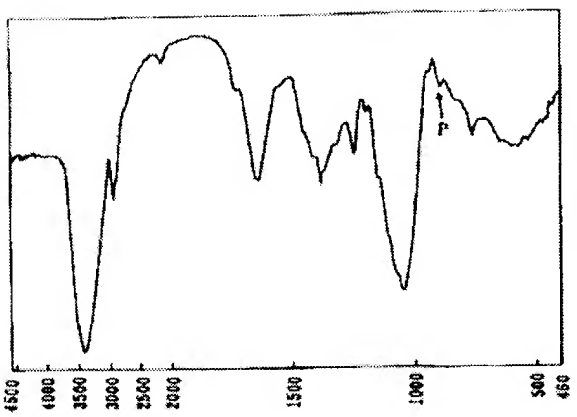


[FIG. 4]

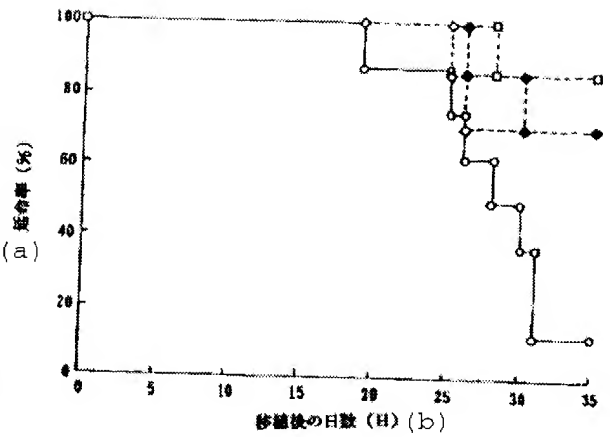


Key: a) DMSO-insoluble component; b) DMSO-soluble component.

[FIG. 5]



[FIG. 6]



Key: a) life-sustaining rate; b) number of days after the transplantation (day).